EXPANDING TEST METHODS FOR MARINE ECOTOXICOLOGY TESTING USING *PROALES SIMILIS*

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EXPANDING TEST METHODS FOR MARINE ECOTOXICOLOGY TESTING USING *PROALES SIMILIS*

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ABSTRACT

This study is the first inquiry into the suitability of the rotifer *Proales similis* as a test animal for researchers studying marine pollution. It also introduces a novel toxicity test based on inhibition of hatching rates of *P. similis* diapausing embryos. We exposed *P. similis* to various concentrations of cadmium, copper, and mercury and then quantified effects on survival, hatching, reproduction, and ingestion. We found that mortality and cyst hatching endpoints were more robust to heavy metal toxicity than reproduction or ingestion. Overall, we confirmed that *P. similis* is a suitable tool for convenient and quick ecotoxicology assessments, and we established cyst inhibition as a novel, reliable endpoint for observing effects of toxicity on rotifer populations.

CHAPTER 1

INTRODUCTION

Previous studies have indicated that a variety of environmental pollutants tend to accumulate in aquatic environments as a result of human activity, such as agricultural and industrial processes (Barth et al. 2007; Bueno et al. 2012). Water systems are highly susceptible to degradation from different sources of pollution, which can wreak havoc on aquatic ecosystems. Ecotoxicologists use model organisms, such as rotifers, to understand how pollution affects the viability of organisms living in aquatic ecosystems. Rotifers are a group of microscopic aquatic invertebrates in the phylum Rotifera and are commonly used in aquatic ecotoxicology studies to evaluate how toxicants can impact ecosystems. The phylum Rotifera is composed of three classes: Seisonidea, Bdelloidea, and Monogonota. Monogonont rotifer populations, which often live in environments that fluctuate in habitability, possess the ability to enter a long-term dormancy by producing stress-resistant diapausing eggs, which are eggs that can remain viable after periods of complete desiccation.

Rotifers are well-established as a tool for ecotoxicology studies due to their short life cycle and simple methods of cultivation. These animals serve a significant role in ecosystems as microinvertebrate zooplankton, so suppression of their population growth can have devastating effects for organisms that depend on them for food. Toxicity assays involving these animals have been used to survey the effects of a variety of toxicants such as heavy metals and pesticides (Snell and Marcial and refs. therein 2017). One rotifer class, Monogononta, often lives in environments that fluctuate in habitability. These rotifers possess the ability to enter a long-term dormancy by producing stress-resistant diapausing eggs that can remain viable after periods of

complete desiccation. Ecotoxicologists have used rotifer acute toxicity assays to extensively study heavy metals used in industrial processes such as copper due to their high toxicity (Janssen et al. 1993; Gama-Flores 2007). In particular, the use of assays with desiccated rotifer diapause eggs is advantageous because it eliminates the need for maintaining live cultures to produce animals.

While rotifers serve as a reliable model organism for assaying toxicants, it remains important to ensure diversity within rotifers used. Developing toxicity tests with new species of rotifers is advantageous for understanding effects of a toxicant on an ecosystem because certain species may be more representative of certain environments. Furthermore, sensitivity to different classes of toxicants varies across different organisms due to their physiology and ecology. *Proales similis* is an attractive animal for developing new methods of toxicity screening in marine habitats because of its different physiology and small size. Their small size may pose an advantage because of greater sensitivity to toxicants at a lower dose. This study hypothesizes that *P. similis* will serve as a reliable bioindicator of toxicity, and it aims to develop a means of estimating the toxic effects of heavy metals in marine waters with a novel rotifer species.

To establish *P. similis* as a dependable test animal, we measured the effect of toxicant exposure on survival, reproduction, ingestion, and hatching. We used cadmium, copper, and mercury as the toxicants surveyed because these heavy metals have been extensively studied with rotifer assays. Thus, researchers would easily be able to compare median lethal concentrations (LC50s) and half-maximal effective concentrations (EC50s) across species (Gama-Flores et al. 2007).

This is the first study directly assessing suitability of *P. similis* as a test animal for researchers studying marine pollution. The study also introduces new methodologies for

developing a new toxicity test based on change in hatching rates of diapausing embryos of *P. similis* following heavy metal exposure. Expanding test options, both in species and endpoints used, will help researchers understand the far-reaching effects of aquatic ecosystem contamination and elucidate how pollutants can differentially affect a variety of species in an ecosystem.

CHAPTER 2

LITERATURE REVIEW

Rotifers have been used in a wide variety of pollution studies over the years, to assess the harmful effects of heavy metals, pesticides, and nanoparticles (Snell and Marcial 2017). Rotifers are highly favored in toxicology screenings because many species can easily and rapidly be revived from dormancy following rehydration, eliminating the need for continuous cultivation of test animals (Ricci 2001, Snell et al. 2017). However, their ease of cultivation has not encouraged researchers to take advantage of a diverse selection of species. While there is extensive data in the literature on a variety of toxicants, most of this data focuses on brachionid species; *Brachionus plicatilis* and *Brachionus rotundiformis* are popular for saltwater while *Brachionus calyciflorus* is the default test organism for freshwater. Many other experimentally convenient rotifer species exist, but ecotoxicologists do not commonly employ them in their toxicity assays. For example, a review of toxicity data for 12 different pesticides revealed that 10 had only assessed brachionid species (Moreira et al 2016). The obvious dearth of toxicity data in the literature is a compelling reason to develop toxicity testing methods for other species. Therefore, adding novel rotifer species to the repertoire of ecotoxicologists is crucial for researchers to understand the full environmental impact of human activity and to know how to organize remediation efforts.

Pesticides are common toxicants that arise from human activities and have been extensively tested using rotifer species. Rotifers generally have a lower sensitivity to insecticides overall but are more sensitive to fungicides when compared to other invertebrates typically used in toxicity studies (Moreira et al. 2016). Other studies have expanded the classes of pollutants

studied using rotifers. Snell and Hicks (2011) successfully investigated the toxic effects of nanoparticles on *Brachionus manjavacas*. Nanoparticles are becoming increasingly common in industrial manufacturing and eventually make their way into water systems after disposal. This study found that while 37 nm diameter particles at a concentration of 0.3 μg/L suppressed population growth rate by 50%, larger particles did not reduce population growth or penetrate the walls of the gut. Unlike toxic metals or pesticides, the nanoparticles studied were chemically inert, so their suppression of population growth rate was directly due to their size and mechanical effects on tissue. These findings illustrate that human industries must not only consider chemical pollution when disposing of waste, but also particulate waste.

Rotifers have also been used to estimate the effects of acute environmental disasters. Rico-Martinez et al. (2013) investigated the effects of the 2010 Macondo oil spill in the Gulf of Mexico by carrying out mortality, reproduction, and cyst inhibition assays with Macondo oil and Corexit 9500A(®), which is an oil dispersant that humans applied to help clean-up efforts. They surveyed the chemicals independently and mixed them in different ratios that represented the maximum exposure concentrations, recommended ratios, and actual ratios used in the spill. Their results revealed that the oil dispersant and the oil itself had similar toxicities when assayed independently of each other, but mortality effects increased 52-fold when mixed, suggesting that initial efforts to quantify environmental impact from the spill may have underestimated the damage caused by these pollutants. When assayed alone, an oil fraction of 11.02% inhibited survival by 50%, while a 2.55% oil fraction inhibited reproduction by 50%, indicating that mortality is about 4 times more resistant to toxicant effects. Cyst inhibition assays show a similar sensitivity to reproduction, with a 2.6% fraction of oil inhibited cyst hatching by 50%; this

finding is significant because new rotifer populations are established from cysts hatching each year.

The suitability of rotifers for toxicity assays has long been recognized due to their small size, high population growth rate, and rapid life cycle (American Society for Testing Materials 1998; Snell and Janssen 1995). These characteristics allow researchers to culture them in standard laboratory settings with little maintenance. Depending on the species, rotifers are stable while desiccated for up to several decades. The oft-studied *Brachionus manjavacas,* for example, can enter a long-term diapause by producing dormant embryos that are resistant to full desiccation and freezing temperatures, but can hatch at high rates within about 24 hours upon rehydration, making them convenient for laboratory storage and usage (Ricci 2001). Research has shown that other rotifers, such as *Philodina* sp., can also enter a long-term dormancy when desiccated (anhydrobiosis), but they re-emerge in their original adult states instead of their offspring hatching after only a few minutes after rehydration, even after years of desiccation (Snell et al. 2017). Because of these unique life cycle characteristics, researchers can rehydrate a small quantity of brachionid resting eggs or a tube of desiccated bdelloids rather than keeping populations as live cultures by serial dilution. As a result, many of the contemporary toxicity assays are developed with newly rehydrated animals in mind.

The convenience of cyst-bases assays has encouraged the use of just one cyst-producing genus, *Brachionus*. Conducting toxicity assays using a diverse range of species is advantageous because some species may be more representative of certain environments, and there is much inter-specific variability in toxicant sensitivity. Some of these differences in sensitivity may arise from adaptations in the natural environment. Rico-Martinez et. al (2013) discovered that, in a series of toxicity screens for Macondo oil and Corexit 9500A(®) with various species of the

Brachionus plicatilis species complex, the *Brachionus* sp. Veracruz isolate was the most resistant to oil. They speculate that because this population lives in the Gulf of Mexico, it may have adapted to natural petroleum leaks.

Diversity in toxicity test species may also be advantageous due to environment-specific characteristics of the habitat in question. In another study, Arias-Almedia and Rico Martinez (2011) conducted acute toxicity screenings for cadmium, lead, mercury, and methyl parathion with the freshwater rotifer *Euchlanis dilatata*. They found that while *E. dilatata* was more sensitive to these toxicants than most other rotifer species, including those of the genus *Brachionus*, it was less sensitive than the crustacean *Daphnia magna*, which is another microinvertebrate used in toxicity assays Furthermore, they pointed out that *E. dilatata* is as a good candidate for researchers who want to focus on toxicants that tend to settle in sediments because it tends to inhabit sediment surfaces, compared to the planktonic *B. calyciflorus* (Arias-Almedia and Rico-Martinez 2011). Naturally, this need for diversity in testing methods has not gone unnoticed. Snell (2000) asserts that developing testing methodologies for alternate species of rotifers is important because toxicity should be assessed with the fastest and least expensive test available over a wide variety of species.

While many species may be good candidates for ecotoxicology studies, *Proales similis* in particular has been a promising candidate. Wullur et al. (2009) has found that it averages about 38.1% shorter and 60.3% narrower in size than the popularly studied *Brachionus rotundiformis*, suggesting that it may potentially have different sensitivities to toxicants compared to rotifers in common use. Furthermore, Hagiwara et al. (2014) has demonstrated that *P. similis* has been successfully mass cultured in commercial aquaculture, with specific utility in feeding fish larvae. While in a well-studied class, its life history and physiology are markedly distinct in ways that

indicate it may also differ in toxicity outcomes. Its body lacks a lorica, making it more flexible compared to other rotifers. Furthermore, it is able to tolerate a high range of salinities, surviving from 2-68 ppt. Its euryhaline nature makes it a versatile candidate for studying pollution in a wide variety of environments; it has been discovered naturally in freshwater, marine, estuarine, brackish, and saline environments (Reyes et al. 2017). It mostly inhabits estuarine and coastal marine environments, where high levels of mercury pollution have been recorded (Jara-Martini 2011). Rebolledo et al. (2018) endorse the use of *P. similis* as a test animal due to its higher sensitivity to mercury compared to *B. plicatilis*, rapid growth rate, and life cycle characteristics. *P. similis* can also survive desiccation via diapausing embryos, eliminating the need to maintain them in live cultures for experiments.

This project takes advantage of the unique physiological and life history traits of *P. similis* to develop protocols that expand options for toxicity testing in marine environments. This is the first study that describes testing methods for *P. similis* in the context of surveying environmental pollution. This expansion of testing options may aid in conservation efforts and insight into how to better manage pollution to reduce impact on marine ecosystems.

CHAPTER 3

METHODS

Proales similis Cultures

Populations of *P. similis* were maintained in serial dilution cultures in 250 mL flasks in 200 mL 15 ppt artificial salt water (ASW) and 10% *Tetraselmis suecica*. Flasks were fed weekly with *T. suecica* with growth medium removed to maintain approximately $2x10^5$ cells/mL. Cultures were covered lightly with aluminum foil to decrease chance of contamination while maintaining oxygen exchange and kept at 25°C under constant illumination.

In order to feed *P. similis*, the motile green alga *Tetraselmis suecica* was grown in 15 ppt modified F Media (Guillard 1983). *T. suecica* was grown in serial dilution cultures in 5 L plastic bags under constant fluorescent illumination, constant aeration, and at 25°C.

Preparation of Desiccated P. similis Tubes

Once the population of a serial culture was at a density of at least 100 animals/mL, it was fed concentrated *T.* suecica and 500 uL aliquots were added to several 0.6 mL microcentrifuge tubes. Tubes were left open to condense the medium by evaporation at 22 °C. Gradual condensation allowed time for *P. similis* to deposit diapausing resting eggs as environmental conditions within the tube became uninhabitable. When approximately 50 uL of brine was left, the tubes were capped. At this volume, salinity of the brine was about 100 ppt and no active animals were observed, leaving behind only the diapausing resting eggs. These diapausing eggs were hatched by adding 500 uL of 15 ppt ASW to each tube and incubating the rehydrated tubes at 22 °C under constant fluorescent illumination. Neonates were hatched and ready to use in

experiments by 18-21 hours. These tubes were stored at 4 ℃ and could be hatched after several months of dormancy.

Preparation of Metal Solutions

Stocks were prepared in 100 mL deionized water with 1 mg/mL concentrations of copper (cupric sulfate), cadmium (cadmium chloride), and mercury (mercury (II) chloride). Stock solutions were diluted in 15 ppt ASW to achieve experimental concentrations.

Toxicity Tests

Toxicity screens were conducted to measure the effects of cadmium, copper, and mercury on the endpoints of survival, population growth rate, ingestion, and diapausing egg hatching on *P. similis*. Initially, range-finding tests were conducted to narrow down the range of metal concentrations at which a linear dose response was observed. The ranges were as follows: 0.09-0.9 mg/L for copper, 0.08-225 mg/L for cadmium, and 0.005-0.35 mg/L for mercury. Each test was replicated three times to find LC50s for acute tests and EC50s for reproductive, ingestion, and diapausing egg hatching tests.

Acute Toxicity Test

Acute toxicity tests were administered to measure survival in response to high cadmium, copper, and mercury concentrations. We used a 25 um filter to extract adult *P. similis* from an established flask population and placed 10 individuals into each well of a 24 well plate. Each well also contained 1 mL 15 ppt artificial salt water (ASW) and the corresponding metal concentration. Each experimental well plate contained either cadmium, copper, or mercury.

Within each well plate, there was a control that had no metal solution and 5 different concentrations of metal, and 4 replicates per concentration. Each plate was incubated at 25 ℃ with no illumination for 6 hours, and then the number of live and dead rotifers were counted and the percent survival and LC50 calculated from that data.

Reproductive Test

Reproductive tests were conducted to estimate population growth rates in response to different metal concentrations. 2 neonate *P. similis* rehydrated from tubes were placed into each well of a 48 well plate containing 500 uL of 15 ppt ASW, 1 x 10^5 cells/mL *T. suecica*, and the appropriate metal concentration. The wells contained algae to ensure that reproductive inhibition was not due to the effects of starvation. Each experimental well plate contained either cadmium, copper, or mercury. Within each well plate, there was a control that had no metal solution and 5 different concentrations of metal, and 8 replicates per concentration. Each plate was incubated at 25 ℃ with no illumination for 72 hours, after which the total number of animals in each well were noted. The equation $r = (\ln(N_t)-\ln(N_0))/T$ was derived for exponential population growth rate and used to calculate average population growth rate, in which r = offspring per rotifer per day, N_t = total number of animals in well after 3 days, N_0 = initial animals in well (2), and T = time (3 days).

Ingestion Test

Ingestion tests were administered to measure feeding response to different cadmium, copper, and mercury concentrations, and were also an assessment of health. 10 adult *P. similis* were filtered with a 25 um filter from an established flask population and placed into each well

of a 24 well plate. Each well also contained 500 mL 15 ppt artificial salt water (ASW) and the corresponding metal concentration. Each experimental well plate contained either cadmium, copper, or mercury. Within each well plate, there was a control that had no metal solution and 5 different concentrations of metal, and 4 replicates per concentration. Each plate was incubated at 25 ℃ with no illumination or algae for 1 hour to ensure hunger, and then 30 uL of a red carmine particle suspension (1 mg/mL in 15 ppt ASW) was added to each well and mixed by gentle agitation. Rotifers ingest carmine in lieu of their normal food when no algae is present, and the accumulation of the pigment is easily visible under 25X magnification (Snell 2005). Each plate was then incubated in the same conditions for an additional 2 hours, and then the number of clear gut (not feeding) and red gut (feeding) rotifers were counted and the percent actively ingesting and EC50 calculated from that data.

Diapausing Egg Hatching Test

Hatching inhibition tests were administered to measure hatching in response to different cadmium, copper, and mercury concentrations. For each metal, 24 *P. similis* tubes were hydrated with 500 mL of 15 ppt ASW containing the appropriate metal concentrations. Each experimental set of tubes contained either cadmium, copper, or mercury. Within each set, there was a control that had no metal solution and 5 different concentrations of metal, and there were 4 replicates per metal concentration. Each set of tubes were incubated at 25 ℃ under constant fluorescent illumination for 24 hours, after which the liquid in each tube was transferred to a well of a 24 well plate. Then, each tube was washed with 250 uL of 15 ppt ASW to capture any remaining animals, and this wash was added to the same well. Based on an initial examination of well

density, the number of hatched rotifers in each well was counted either directly from the well or estimated from 4 subsamples of 10-30 uL from the well when direct counts were not feasible.

Statistical Analysis

Dose-response linear regressions were calculated based on endpoint data using Microsoft Excel. LC50s, EC50s, coefficients of variation and 95% confidence intervals were calculated based on the regressions.

CHAPTER 4

RESULTS

Cadmium Toxicity

Cadmium was the least toxic to *P. similis.* With cadmium exposure, reproduction over 72 hours was most inhibited, yielding the lowest EC50 value of 1.01 mg Cd/L. The next most sensitive endpoint was 3 hour ingestion (EC50 = 2.2 mg Cd/L), then egg hatching (EC50 = 31.3 mg Cd/L). Mortality was the least affected, yielding the highest EC50 value of 50.1 mg Cd/L. Overall, dose response followed a linear relationship, with R^2 values ranging from 0.89-0.99.

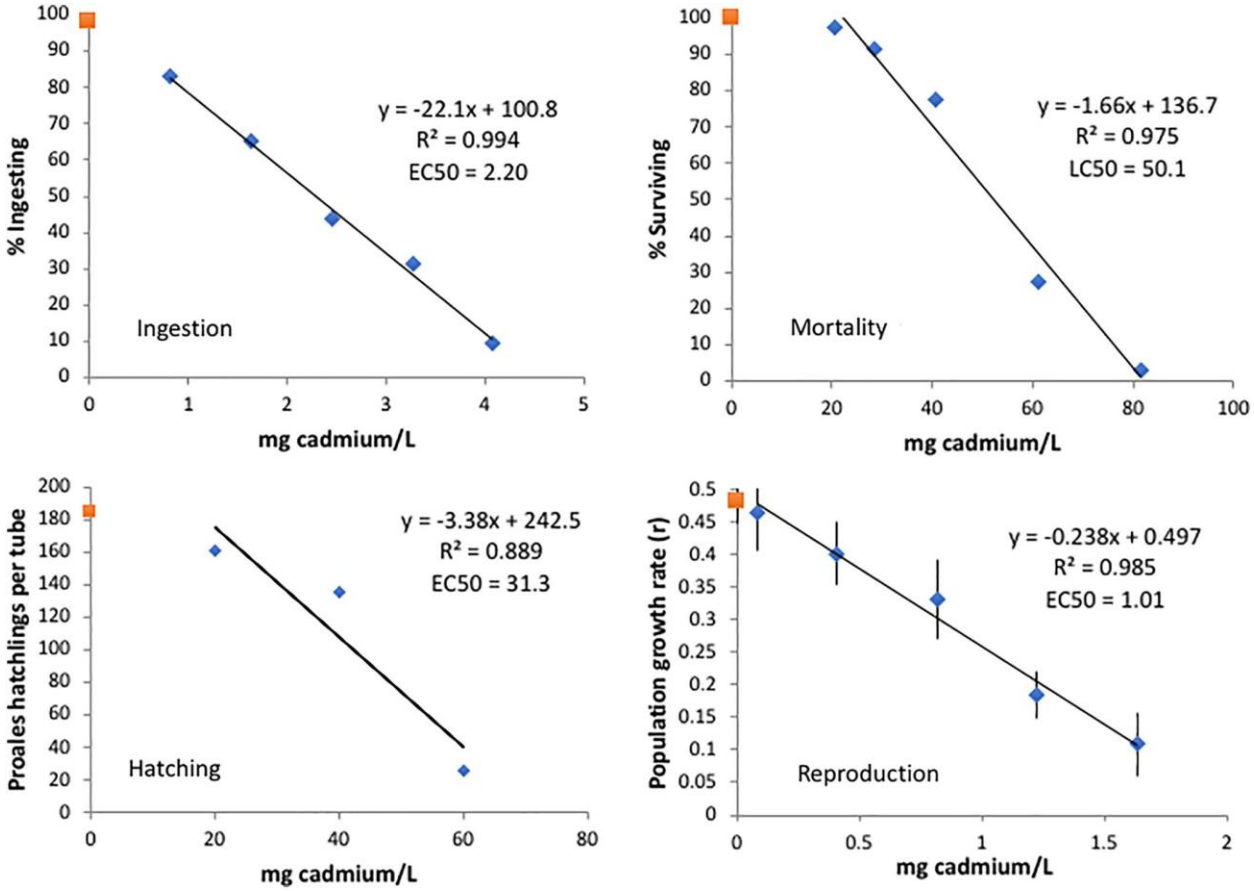

Figure 1. Linear dose response of ingestion, mortality, hatching, and reproduction to cadmium. The EC50s and LC50s are the mean of 3 replicate experiments, but data is shown from only 1 of the replicates each. The orange square on the y-axis indicates the control while the blue diamonds indicate data points from the cadmium treatments. Vertical lines on the reproduction plot represent standard error.

Copper Toxicity

Copper had intermediate toxicity to *P. similis* of the 3 heavy metals surveyed. With copper exposure, reproduction over 72 hours was the most inhibited, yielding the lowest EC50 value of 0.15 mg Cu/L. The next most sensitive endpoint was 3 hour ingestion (EC50 = 0.26 mg Cu/L), and then acute survival (EC50 = 1.06 mg Cu/L). Hatching was the least affected, yielding the highest EC50 value of 3.4 mg Cu/L. Overall, dose response followed a linear relationship, with R^2 values ranging from 0.93-0.98.

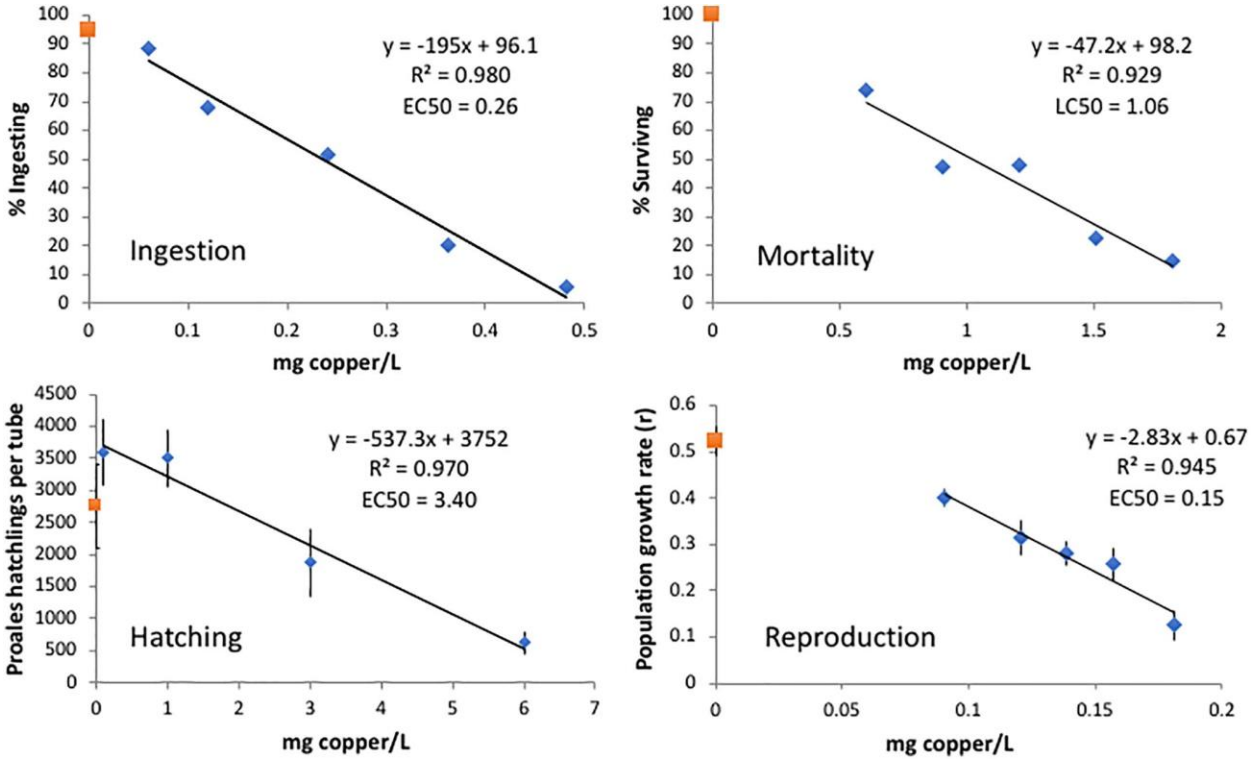

Figure 2. Linear dose response of ingestion, mortality, hatching, and reproduction to copper. The EC50s and LC50s are the mean of 3 replicate experiments, but data is shown from only 1 of the replicates each. The orange square on the y-axis indicates the control while the blue diamonds indicate data points from the copper treatments. Vertical lines on the reproduction plot represent standard error.

Mercury Toxicity

Mercury was the most toxic to *P. similis*. With mercury exposure, ingestion over 3 hours was the most inhibited, yielding the lowest EC50 value of 11 µg Hg/L. The next most sensitive endpoint was 6 hour mortality (EC50 = 26 µg Hg/L), followed by 72 hour reproduction (EC50 = 52 µg Hg/L). Hatching was the least affected, yielding the highest EC50 value of 101 µg Hg/L. Overall, dose response followed a linear relationship, with R^2 values ranging from 0.82-0.99.

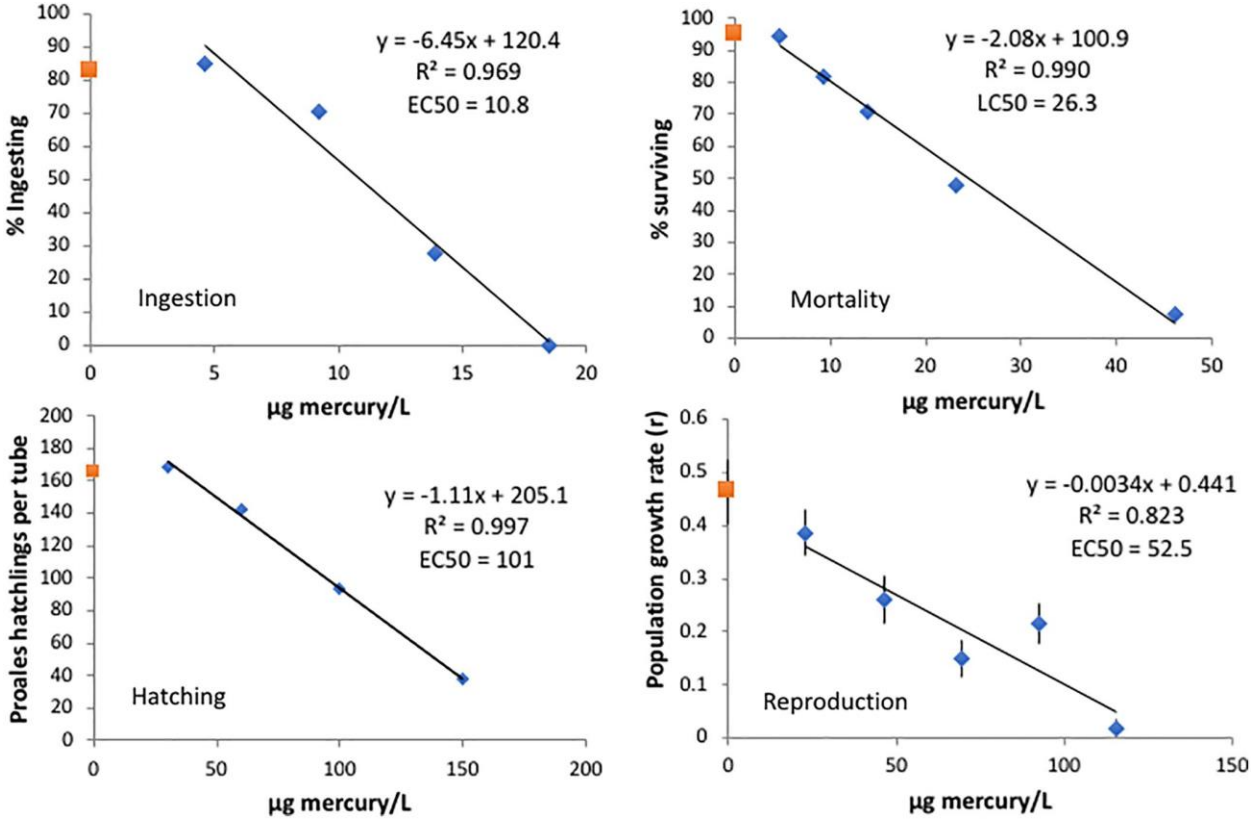

Figure 3. Linear dose response of ingestion, mortality, hatching, and reproduction to mercury The EC50s and LC50s are the mean of 3 replicate experiments, but data is shown from only 1 of the replicates each. The orange square on the y-axis indicates the control while the blue diamonds indicate data points from the mercury treatments. Vertical lines on the reproduction plot represent standard error.

Summary

The mean EC50s, number of replicates, standard deviations, coefficients of variation, and 95% confidence limits for all endpoints and metals are summarized in Table 1.

Metal	Endpoint	N	Mean (mg/L)	Coef. Var.	Upper 95% CL	Lower 95% CL
Cadmium	Mortality (LC50)	3	50.09	3.67	52.17	48.01
	Reproduction (EC50)	3	1.01	6.49	1.08	0.94
	Ingestion (EC50)	3	2.20	13.23	2.53	1.87
	Hatching (EC50)	7	31.29	35.97	46.90	15.71
Copper	Mortality (LC50)	3	1.06	3.69	1.11	1.02
	Reproduction (EC50)	3	0.15	4.68	0.16	0.14
	Ingestion (EC50)	3	0.26	2.74	0.28	0.24
	Hatching (EC50)	3	3.40	19.5	4.64	2.15
Mercury	Mortality (LC50)	3	0.026	2.85	0.027	0.025
	Reproduction (EC50)	3	0.052	18.62	0.064	0.041
	Ingestion (EC50)	3	0.011	11.56	0.012	0.009
	Hatching	4	0.101	39.1	0.149	0.052

Table 1. Summary of toxicity endpoints (LC50s and EC50s) for heavy metals (copper, mercury, cadmium).

CHAPTER 5

DISCUSSION

Higher concentrations of metals inhibited hatching, demonstrating that tracking hatching rates of *Proales similis* can be an effective estimator of metal pollution in marine environments. The endpoint of hatching, along with mortality, reproduction, and ingestion, can serve as practical tools for those who want to use rotifers as bioindicators of toxicity in marine habitats. Overall, mortality and resting egg rates tended to be the most resistant to heavy metal toxicity (Table 1). Robustness of mortality is crucial when considering the local extinction of an active population of *P. similis*, as high acute mortality rates prevent the production of diapause eggs that can persist throughout unfavorable conditions. Furthermore, the commencement of a new population after rehydration of a dormant population is governed by the number and viability of diapausing eggs. In contrast, reproduction and ingestion rates were more sensitive to heavy metal toxicity (Table 1). Inhibition of reproduction hinders the population growth rate, leading to an endangerment of the population in question over a longer time scale, especially when considering the reduced production of diapause eggs. Feeding behavior is significant because of its direct implications on the survival and reproduction. Overall, we demonstrated that *P. similis* can be used by ecotoxicologists for marine toxicity assays to expand the diversity of animals used in such screenings.

We also established diapause egg hatching inhibition rates as a suitable endpoint for observing toxicity within a species. However, hatching inhibition also had the highest standard deviation across all surveyed heavy metals, which may be improved by reducing variation among the condensed egg tubes. Diapausing egg hatching is an important measure of population

health because they are crucial for ensuring the long-term viability of a population and re-establishing a population after a period of stress. Furthermore, eggs tend to settle in sediments, so studying hatching inhibition may be more relevant when assessing the ecosystem effects of toxicants that also tend to settle in sediment rather than circulating in the water column (Rico-Martinez et al. 2013). The methods outlined for assessing hatching inhibition could also be adapted to *Brachionus* strains as well when considering toxicants that occupy areas lower in the water column.

CHAPTER 6

CONCLUSION

Certain species may serve as a more accurate representation of certain environments. In particular, *P. similis* inhabits coastal marine habitats where heavy metal accumulation poses a problem. Furthermore, endpoints differ in sensitivity to different toxicant classes. *P. similis* is a valuable tool for toxicity screening in marine habitats because of its small size, toxicant sensitivity, and production of diapausing eggs. In addition, *P. similis* is a convenient and cost-effective species to use in toxicity assays, as their rapid hatching (<24 hours) enables researchers to hydrate diapause eggs only when needed, and we have demonstrated that it can be reliably used for toxicity tests. Our findings will allow ecotoxicologists to expand the repertoire of animals used in toxicity assays so that toxicity screens can survey a wider variety of animals. Evaluating how toxicant exposure inhibits diapause egg hatching is also a compelling new method of trying to understand the far-reaching effects of toxicant exposure in aquatic ecosystems.

CHAPTER 7

FUTURE WORK

We have demonstrated the viability of *P. similis* diapausing eggs, and successfully outlined methods to collect them in tubes. However, we have yet to elucidate the mechanism by which they are produced. *Brachionus* species employ cyclical parthenogenesis, and they exhibit sexual dimorphism, but we have yet to observe males or sexual reproduction in our *P. similis* populations. Furthermore, we have not been able to distinguish between stress-resistant eggs and directly developing eggs through microscopic analysis or lipid staining. We are therefore unsure if two different types of eggs are produced in its life cycle or if diapause eggs are simply highly variable. We are interested in investigating alternate methods of analyzing eggs in order to illuminate the genetic basis of the life cycle of *P. similis.*

With *P. similis,* we have only surveyed the effects of 3 heavy metals, so there is a wealth of potential for studying variations in toxicant sensitivity for other compounds, such as pesticides or nanoparticles. Furthermore, there is a huge diversity of rotifers which have not been investigated for potential use in toxicity assays. Future work in this field will address hatching rates of other rotifer species when exposed to toxicants. We are interested in exploring various other monogonont species living in diverse environments that can be developed for cyst-based toxicity tests.

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